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PTO/SB/05 (4/98)

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No. **HS 102 DIV**
 First Inventor or Application Identifier **Gary A. Bannon**
 Title **METHODS AND REAGENTS FOR DECREASING
CLINICAL REACTION TO ALLERGY**
 Express Mail Label No. **EL320554231US**

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ☒ Specification [Total Pages **35**]
(preferred arrangement set forth below)
 - Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets **3**]
4. Oath or Declaration [Total Pages **5**]
 - a. ☐ Newly executed (original or copy)
 - b. ☒ Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 16 completed)
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

* NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

5. ☐ Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☒ Paper Copy (identical to computer copy)
 - c. ☒ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment Papers (cover sheet & document(s))
8. ☒ 37 C.F.R. § 3.73(b) Statement (when there is an assignee) ☒ Power of Attorney
9. ☐ English Translation Document (if applicable)
10. ☒ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
11. ☒ Preliminary Amendment
12. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
13. ☒ * Small Entity Statement(s) ☒ Statement filed in prior application, Status still proper and desired (PTO/SB/09-12)
14. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
15. ☒ Other: Check for \$407.00

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No: **09 / 141,220**
 Prior application information: Examiner **Nolan, P.** Group / Art Unit: **1644**

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label or ☒ Correspondence address below
 (Insert Customer No. or Attach bar code label here)

Name	Patrea L. Pabst, Esq.				
	Arnall Golden & Gregory, LLP				
Address	2800 One Atlantic Center				
	1201 West Peachtree Street				
City	Atlanta	State	GA	Zip Code	30309-3450
Country	United States	Telephone	(404) 873-8794	Fax	(404) 873-9795

Name (Print/Type)	Patrea L. Pabst	Registration No. (Attorney/Agent)	31,284
Signature		Date	11/6/00

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

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jc511 U.S. PTO
 09/4/8668

01/06/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin

Serial No.: Divisional of U.S.S.N. 09/141,220

Express Mail Label
No.: EL320554231US

Filed: January 6, 2000

Date of Deposit: January 6, 2000

For: *METHODS AND REAGENTS FOR DECREASING CLINICAL
REACTION TO ALLERGY*

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

**REQUEST FOR FILING A DIVISIONAL APPLICATION
UNDER 37 C.F.R. § 1.53(b)**

Sir:

Pursuant to 35 U.S.C. § 21(a), as amended by Public Law 97-247, and 37 C.F.R. §§ 1.10 and 1.53(b), Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin enclose for filing the attached new divisional patent application entitled "*Methods and Reagents for Decreasing Clinical Reaction to Allergy*". The application includes a copy of prior application U.S.S.N. 09/141,220, including 1 page of abstract, 29 pages of specification, and 5 pages of claims. This application is a divisional of pending prior application Serial No. 09/141,220, filed August 27, 1998, entitled "*Methods and Reagents for Decreasing Clinical Reaction to Allergy*", by Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin.

Pursuant to 37 C.F.R. § 1.63(d), the copy of the executed Declaration included in the new application is a copy of the executed Substitute Declaration filed September 2, 1999, in parent application Serial No. 09/141,220, to which the new application claims benefit.

Please preliminarily amend the application in accordance with the enclosed Preliminary Amendment. An Information Disclosure Statement is also enclosed.

Enclosed from prior application U.S.S.N. 09/141,220 are three sheets of formal drawings; copies of (1) Statement Claiming Small Entity Status by assignee University of Arkansas; (2) Statement Under 37 C.F.R. § 3.73(b) by assignee University of Arkansas; (3) Assignment from A. Wesley Burks, Jr. and Gary A. Bannon to University of Arkansas, recorded at Reel 010065, Frame 0008; (4) Statement Claiming Small Entity Status by assignee Mt. Sinai School of Medicine of the City University of New York; (5) Statement Under 37 C.F.R. § 3.73(b) by assignee Mt. Sinai School of Medicine of the City University of New York; (6) Assignment from Hugh A. Sampson to Mt. Sinai School of Medicine of the City University of New York, recorded at Reel 009539, Frame 0550; (7) Statement Claiming Small Entity Status by assignee Panacea Pharmaceuticals, LLC; (8) Statement Under 37 C.F.R. § 3.73(b) by assignee Panacea Pharmaceuticals, LLC.; and (9) Assignment from Howard Sosin to Panacea Pharmaceuticals, Inc., recorded at Reel 010190, Frame 0516.

A check in the amount of \$407.00 to cover the \$345.00 filing fee for a small entity and the \$27.00 fee for three claims in excess of twenty total claims is enclosed. The Commissioner is authorized to credit Deposit Account No. 01-2507 in the amount of \$35.00, which represents

Divisional of U.S.S.N. 09/141,220
"Methods and Reagents for Decreasing Clinical Reaction to Allergy"
By: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin
**REQUEST FOR FILING A DIVISIONAL
APPLICATION UNDER 37 C.F.R. § 1.53(b)**
Express Mail Label No.: EL320554231US
Date of Deposit: January 6, 2000

the difference between the small entity filing fee effective prior to December 29, 1999, and the current fee. It is believed that this is the proper filing fee since the application includes 3 independent claims and a total of 23 claims after entry of the Preliminary Amendment. This application is entitled to small entity status as copies of the executed Statements Claiming Small Entity Status by each of the three assignees filed in prior application 09/141,220 are enclosed. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 01-2507. To facilitate this process, applicants have enclosed a duplicate of this letter.

The power of attorney in the prior application is to Patrea L. Pabst.

The inventorship for the claims in the new application is the same as in parent application Serial No. 09/141,220.

This application contains nucleic acid and/or protein sequences as defined in 37 C.F.R. § 1.821-1.825. The sequence listing for the new application is identical to the sequence listing for application Serial No. 09/141,220, filed August 27, 1998, by Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin. Sequence listings in computer readable form were submitted in Application Serial No. 09/141/220 on August 27, 1998, and again on December 10, 1999 in response to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures. Accordingly, pursuant to 37 C.F.R. § 1.821(e), applicants hereby request that the computer readable form of the sequence listing submitted in application Serial No. 09/141/220 on

Divisional of U.S.S.N. 09/141,220
"Methods and Reagents for Decreasing Clinical Reaction to Allergy"
By: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin
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APPLICATION UNDER 37 C.F.R. § 1.53(b)**
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December 10, 1999, be used as the computer readable form of the sequence listing for the new application. The application has a paper copy of the Sequence Listing incorporated therein.

I declare that the material in the prior sequence listing is identical to the paper copy of the Sequence Listing present in the new application, that the Sequence Listing does not add new matter to the application, and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This application is being filed on January 6, 2000, by mailing the application to the Assistant Commissioner of Patents, Washington, D.C. 20231 via the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 C.F.R. § 1.10. The Express Mail Label Number appears in the heading of this paper which is attached to the application papers pursuant to 37 C.F.R. § 1.10(b).

All correspondence concerning this application should be mailed to:

Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, GA 30309-3450

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the

Divisional of U.S.S.N. 09/141,220

"Methods and Reagents for Decreasing Clinical Reaction to Allergy"

By: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin

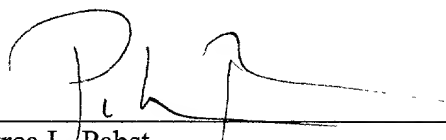
**REQUEST FOR FILING A DIVISIONAL
APPLICATION UNDER 37 C.F.R. § 1.53(b)**

Express Mail Label No.: EL320554231US

Date of Deposit: January 6, 2000

United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,



Patrea L. Pabst
Reg. No. 31,284

Date: January 6, 2000

ARNALL, GOLDEN & GREGORY, LLP
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Atlanta, Georgia 30309-3450
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(404) 873-8795 (fax)

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**STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(d))--NONPROFIT ORGANIZATION**

Docket Number (Optional)
HS 102

Applicant, Patentee, or Identifier: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin

Application or Patent No.: 09/141,220

Filed or Issued: August 27, 1998

Title: METHODS AND REAGENTS FOR DECREASING
CLINICAL REACTION TO ALLERGY

I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF NONPROFIT ORGANIZATION The University of Arkansas

ADDRESS OF NONPROFIT ORGANIZATION 2404 North University Avenue
Little Rock, Arkansas 72207-3608

TYPE OF NONPROFIT ORGANIZATION:

☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))

☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))
IF LOCATED IN THE UNITED STATES OF AMERICA

☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED
STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby state that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

- ☐ the specification filed herewith with title as listed above.
☒ the application identified above.
☐ the patent identified above.

I hereby state that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern, or organization having rights in the invention must file separate statements as to their status as small entities and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern, or organization having any rights in the invention is listed below:

- ☐ no such person, concern, or organization exists.
☒ each such person, concern, or organization is listed below.

Mt. Sinai School of Medicine of the City University of New York, One Gustave L. Levy Place,

New York, NY 10029-6574; Panacea Pharmaceuticals, LLC, 640 Sasco Hill Road, Fairfield, Connecticut 06430

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

NAME OF PERSON SIGNING _____

Mr. Harold J. Evans

TITLE IN ORGANIZATION OF PERSON SIGNING _____

Assoc. V.P. For Legal Affairs

ADDRESS OF PERSON SIGNING _____

University of Arkansas

2404 North University Avenue

Little Rock, AR 72207

SIGNATURE [Signature]

DATE November 9, 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin

Serial No.: Divisional of U.S.S.N. 09/141,220

Express Mail Label
No.: EL320554231US

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For: *METHODS AND REAGENTS FOR DECREASING CLINICAL
REACTION TO ALLERGY*

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examination of the above-identified divisional application, please amend the application as follows.

In the Specification

On page 1, line 6, please delete "Priority is claimed" and insert in place thereof

--This is a divisional of Serial No. 09/141,220, filed August 27, 1998, which claims priority--.

Divisional of U.S.S.N. 09/141,220

"Methods and Reagents for Decreasing Clinical Reaction to Allergy"

By: Gary A. Bannon, A. Wesley Burks, Jr., Hugh A. Sampson, and Howard B. Sosin

PRELIMINARY AMENDMENT

Express Mail Label No.: EL320554231US

Date of Deposit: January 6, 2000

In the Claims

Please cancel claims 1-13.

Respectfully submitted,



Patrea L. Pabst
Reg. No. 31,284

Date: January 6, 2000

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APPLICATION
FOR
UNITED STATES LETTERS PATENT

BY

GARY A. BANNON

A. WESLEY BURKS

HUGH SAMPSON

AND

HOWARD SOSIN

FOR
METHODS AND REAGENTS FOR DECREASING
CLINICAL REACTION TO ALLERGY

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

Background of the Invention

The United States government has rights in this invention by virtue of grants from the National Institute of Health RO1-AI33596.

Priority is claimed to U.S. Serial No. 60/074,590 filed February 13, 1998, entitled "Mutagenized IgE-Binding Epitopes for Ara H 1, by A. Wesley Burks, Jr., Ricki M. Helm, Gael Cockrell, Gary A. Bannon, J. Steven Stanley, and David Shin; U.S. Serial No. 60/074,624 filed February 13, 1998, entitled "Peanut Allergen Ara H 3" by A. Wesley Burks, Jr., Hugh Sampson, Gary A. Bannon, Shau-Ku Huang, and Patrick A. Rabjohn; U.S. Serial No. 60/074633 filed February 13, 1998, entitled "Mutagenized IgE-Binding Epitopes for Ara H 2" by A. Wesley Burks, Jr., Ricki M. Helm, Gael Cockrell, Gary A. Bannon, J. Steven Stanley, and Nina King; and under 35 U.S.C. §§ 120 and 371 to PCT/US96/15222 entitled "Peanut Allergens and Methods" filed September 23, 1996, by University of Arkansas.

Allergic disease is a common health problem affecting humans and companion animals (mainly dogs and cats) alike. Allergies exist to foods, molds, grasses, trees, insects, pets, fleas, ticks and other substances present in the environment. It is estimated that up to 8% of young children and 2% of adults have allergic reactions just to foods alone. Some allergic reactions (especially those to foods and insects) can be so severe as to be life threatening. Problems in animals tend to be less severe, but very common. For example, many dogs and cats have allergies to flea saliva proteins, grasses, and other common substances present in the environment.

Allergy is manifested by the release of histamines and other mediators of inflammation by mast cells which are triggered into action when IgE

antibodies bound to their receptors on the mast cell surface are cross linked by antigen. Other than avoidance, and drugs (e.g. antihistamines, decongestants, and steroids) that only treat symptoms and can have unfortunate side effects and often only provide temporary relief, the only currently medically accepted treatment for allergies is immunotherapy. Immunotherapy involves the repeated injection of allergen extracts, over a period of years, to desensitize a patient to the allergen. Unfortunately, traditional immunotherapy is time consuming, usually involving years of treatment, and often fails to achieve its goal of desensitizing the patient to the allergen. Furthermore, it is not the recommended treatment for food allergies, such as peanut allergies, due to the risk of anaphylaxis.

Noon (Noon, *Lancet* 1911; 1:1572-73) first introduced allergen injection immunotherapy in 1911, a practice based primarily on empiricism with non-standardized extracts of variable quality. More recently the introduction of standardized extracts has made it possible to increase the efficacy of immunotherapy, and double-blind placebo-controlled trials have demonstrated the efficacy of this form of therapy in allergic rhinitis, asthma and bee-sting hypersensitivity (BSAC Working Party, *Clin. Exp. Allergy* 1993; 23:1-44). However, increased risk of anaphylactic reactions has accompanied this increased efficacy. For example, initial trials of immunotherapy to food allergens has demonstrated an unacceptable safety:efficacy ratio (Oppenheimer et al. *J Allergy Clin. Immun.* 1992; 90:256-62; Sampson, *J. Allergy Clin. Immun.* 1992; 90:151-52; Nelson et al. *J. Allergy Clin. Immun.* 1996; 99:744-751). Results like these have prompted investigators to seek alternative forms of immunotherapy as well as to seek other forms of treatment.

Initial trials with allergen-non-specific anti-IgE antibodies to deplete the patient of allergen-specific IgE antibodies have shown early promise (Boulet, et al. 1997; 155:1835-1840; Fahy, et al. *American J Respir. Crit.*

Care Med. 1997; 155:1828-1834; Demoly P. and Bousquet J. *American J Resp. Crit. Care Med.* 1997; 155:1825-1827). On the other hand, trials utilizing immunogenic peptides (representing T cell epitopes) have been disappointing (Norman, et al. *J. Aller. Clin. Immunol.* 1997; 99:S127).
 5 Another form of allergen-specific immunotherapy which utilizes injection of plasmid DNA (Raz et al. *Proc. Nat. Acad. Sci. USA* 1994; 91:9519-9523; Hz et al. *Int. Immunol.* 1996; 8:1405-1411) remains unproven.

There remains a need for a safe and efficacious therapy for allergies, especially those where traditional immunotherapy is ill advised due to risk to
 10 the patient or lack of efficacy. There is also a need for alternatives to therapies, for example, by creating foods, materials or substances that do not include the allergens that are most problematic, or which contain modified allergens which do not elicit the same reaction. While the technology to make genetically engineered plants and animals is at this point well
 15 established, useful modifications would require understanding how allergens can be modified so that they retain the essential functions for the plants' and animals' nutritional value, taste characteristics, etc., but no longer elicit as severe an allergic response.

It is therefore an object of the present invention to provide a method for decreasing the allergenicity of allergens either by modifying the allergen
 20 itself or by producing a compound that would mask the epitope and thus prevent binding of IgE.

It is a further object of the present invention to provide allergens that elicit fewer IgE mediated responses.

25 It is still another object of the present invention to provide a method to make genetically engineered plants and animals that elicit less of an allergic response than the naturally occurring organisms.

Summary of the Invention

It has been determined that allergens, which are characterized by both humoral (IgG and IgE) and cellular (T cell) binding sites, can be made less allergenic by modifying the IgE binding sites. The IgE binding sites can be eliminated by masking the site with a compound that would prevent IgE binding or by altering as little as a single amino acid within the protein to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, (i.e. only within the IgE-binding sites) while retaining the ability of the protein to activate T cells and, optionally, to bind IgG. Binding sites are identified using known techniques, such as by binding with antibodies in pooled sera obtained from individuals known to be immunoreactive with the allergen to be modified. Proteins that are modified to alter IgE binding are screened for binding with IgG and/or activation of T cells.

Peanut allergens (Ara h 1, Ara h 2, and Ara h 3) have been used in the examples to demonstrate alteration of IgE binding sites while retaining binding to IgG and activation of T cells. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding were determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

Standard techniques such as a skin test for wheal and flare formation can be used to assess decreased allergenicity of modified proteins, created as described in the examples. The modified allergens can also be tested for binding to IgG and proliferation of T cells, and modified allergens selected for optimal stimulation of T cells and binding IgG.

The immunotherapeutics can be delivered by standard techniques,

using injection, by aerosol, sublingually, topically (including to a mucosal surface), and by gene therapy (for example, by injection of the gene encoding the immunotherapeutic into muscle or skin where it is transiently expressed for a time sufficient to induce tolerance).

This method and the criteria for identifying and altering allergens can be used to design useful proteins (including nucleotide molecules encoding the proteins) for use in immunotherapy, to make a vaccine and to genetically engineer organisms such as plants and animals which then produce proteins with less likelihood of eliciting an IgE response. Techniques for engineering plants and animals are well known. Based on the information obtained using the method described in the examples, one can engineer plants or animals to cause either site specific mutations in the gene encoding the protein(s) of interest, or to knock out the gene and then insert the gene encoding the modified protein.

Brief Description of the Drawings

Figure 1 shows an example of how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 1 allergen.

Figure 2 shows how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 2 allergen.

Figure 3 shows how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 3 allergen.

Figure 4 shows how amino acids critical to IgE binding were identified.

Figure 5A shows the location of altered residues within the Ara h 2 amino acid sequence (SEQ ID NO 4).

Figure 5B shows the effect the modified Ara h 2 protein has on IgE binding.

Figure 5C shows the effect the modified Ara h 2 protein has on IgG binding.

Figure 6 shows the results of T-cell proliferation assays using the wild-type and modified Ara h 2 protein.

Detailed Description of the Invention

Definitions

The following definitions are used herein.

An antigen is a molecule that elicits production of antibody (a humoral response) or an antigen-specific reaction with T cells (a cellular response).

An allergen is a subset of antigens which elicits IgE production in addition to other isotypes of antibodies.

An allergic reaction is one that is IgE mediated with clinical symptoms primarily involving the cutaneous (urticaria, angiodema, pruritus), respiratory (wheezing, coughing, laryngeal edema, rhinorrhea, watery/itching eyes), gastrointestinal (vomiting, abdominal pain, diarrhea), and cardiovascular (if a systemic reaction occurs) systems.

An epitope is a binding site including an amino acid motif of between approximately six and fifteen amino acids which can be bound by either an immunoglobulin or recognized by a T cell receptor when presented by an antigen presenting cell in conjunction with the major histocompatibility complex (MHC). A linear epitope is one where the amino acids are recognized in the context of a simple linear sequence. A conformational epitope is one where the amino acids are recognized in the context of a particular three dimensional structure.

An immunodominant epitope is one which is bound by antibody in a large percentage of the sensitized population or where the titer of the antibody is high, relative to the percentage or titer of antibody reaction to other epitopes present in the same protein.

A decreased allergic reaction is characterized by a decrease in clinical symptoms following treatment of symptoms associated with exposure to an

allergen, which can involve respiratory, gastrointestinal, skin, eyes, ears and mucosal surfaces in general.

An antigen presenting cell (an APC) is a cell which processes and presents peptides to T cells to elicit an antigen-specific response.

Immunostimulatory sequences are oligodeoxynucleotides of bacterial, viral or invertebrate origin that are taken-up by APCs and activate them to express certain membrane receptors (e.g., B7-1 and B7-2) and secrete various cytokines (e.g., IL-1, IL-6, IL-12, TNF). These oligodeoxynucleotides containing unmethylated CpG motifs cause brisk activation and when injected into animals in conjunction with antigen, appear to skew the immune response to a Th1-type response. See, for example, Yamamoto, et al., *Microbiol. Immunol.* 36, 983 (1992); Krieg, et al., *Nature* 374, 546-548 (1995); Pisetsky, *Immunity* 5, 303 (1996); and Zimmerman, et al., *J. Immunol.* 160, 3627-3630 (1998).

I. Diagnostic and Therapeutic Reagents.

The first step in making the modified allergen is to identify IgE binding sites and/or immunodominant IgE binding sites. The second step is to mutate one or more of the IgE binding sites, preferably including at a minimum one of the immunodominant sites, or to react the allergen with a compound that selectively blocks binding to one or more of the IgE binding sites. The third step is to make sufficient amounts of the modified allergen for administration to persons or animals in need of tolerance to the allergen, where the modified allergen is administered in a dosage and for a time to induce tolerance, or for diagnostic purposes. The modified allergen can be administered by injection, or in some cases, by ingestion or inhalation.

A. Allergens.

Many allergens are known that elicit allergic responses, which may range in severity from mildly irritating to life-threatening. Food allergies are mediated through the interaction of IgE to specific proteins contained within

the food. Examples of common food allergens include proteins from peanuts, milk, grains such as wheat and barley, soybeans, eggs, fish, crustaceans, and mollusks. These account for greater than 90% of the food allergies (Taylor, Food Techn. 39, 146-152 (1992). The IgE binding epitopes from the major allergens of cow milk (Ball, et al. (1994) *Clin. Exp. Allergy*, 24, 758-764), egg (Cooke, S.K. and Sampson, H.R. (1997) *J. Immunol.*, 159, 2026-2032), codfish (Aas, K., and Elsayed, S. (1975) *Dev. Biol. Stand.* 29, 90-98), hazel nut (Elsayed, et al. (1989) *Int. Arch. Allergy Appl. Immunol.* 89, 410-415), peanut (Burks et al., (1997) *Eur. J. Biochemistry*, 245:334-339; Stanley et al., (1997) *Archives of Biochemistry and Biophysics*, 342:244-253), soybean (Herein, et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 92, 193-198) and shrimp (Shanty, et al. (1993) *J. Immunol.* 151, 5354-5363) have all been elucidated, as have others. Other allergens include proteins from insects such as flea, tick, mite, fire ant, cockroach, and bee as well as molds, dust, grasses, trees, weeds, and proteins from mammals including horses, dogs, cats, etc.

The majority of allergens discussed above elicit a reaction when ingested, inhaled, or injected. Allergens can also elicit a reaction based solely on contact with the skin. Latex is a well known example. Latex products are manufactured from a milky fluid derived from the rubber tree, *Hevea brasiliensis* and other processing chemicals. A number of the proteins in latex can cause a range of allergic reactions. Many products contain latex, such as medical supplies and personal protective equipment. Three types of reactions can occur in persons sensitive to latex: irritant contact dermatitis, and immediate systemic hypersensitivity. Additionally, the proteins responsible for the allergic reactions can fasten to the powder of latex gloves. This powder can be inhaled, causing exposure through the lungs. Proteins found in latex that interact with IgE antibodies were characterized by two-dimensional electrophoresis. Protein fractions of 56, 45, 30, 20, 14, and

less than 6.5 kd were detected (Posch A. et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 385-395). Acidic proteins in the 8-14 kd and 22 - 24 kd range that reacted with IgE antibodies were also identified (Posch A. et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 385-395. The proteins prohevein and hevein, from hevea brasiliensis, are known to be major latex allergens and to interact with IgE (Alenius, H., et al., *Clin. Exp. Allergy* 25(7), 659-665; Chen Z., et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 402-409). Most of the IgE binding domains have been shown to be in the hevein domain rather than the domain specific for prohevein (Chen Z., et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 402-409). The main IgE-binding epitope of prohevein is thought to be in the N-terminal, 43 amino acid fragment (Alenius H., et al., (1996) *J. Immunol.* 156(4), 1618-1625). The hevein lectin family of proteins has been shown to have homology with potato lectin and snake venom disintegrins (platelet aggregation inhibitors) (Kielisqewski, M.L., et al., (1994) *Plant J.* 5(6), 849-861).

B. Identification of IgE Binding Sites.

Allergens typically have both IgE and IgG binding sites and are recognized by T cells. The binding sites can be determined either by using phage display libraries to identify conformational epitopes (Eichler and Houghten, (1995) *Molecular Medicine Today* 1, 174-180; Jensen-Jarolim et al., (1997) *J. Appl. Clin. Immunol.* 101, 5153a) or by using defined peptides derived from the known amino acid sequence of an allergen (see examples below), or by binding of whole protein or protein fragments to antibodies, typically antibodies obtained from a pooled patient population known to be allergic to the allergen. It is desirable to modify allergens to diminish binding to IgE while retaining their ability to activate T cells and in some embodiments by not significantly altering or decreasing IgG binding capacity. This requires modification of one or more IgE binding sites in the allergen.

A preferred modified allergen is one that can be used with a majority

of patients having a particular allergy. Use of pooled sera from allergic patients allows determination of one or more immunodominant epitopes in the allergen. Once some or all of the IgE binding sites are known, it is possible to modify the gene encoding the allergen, using site directed mutagenesis by any of a number of techniques, to produce a modified allergen as described below, and thereby express modified allergens. It is also possible to react the allergen with a compound that achieves the same result as the selective mutation, by making the IgE binding sites inaccessible, but not preventing the modified allergen from activating T cells, and, in some embodiments, by not significantly altering or decreasing IgG binding.

Assays to assess an immunologic change after the administration of the modified allergen are known to those skilled in the art. Conventional assays include RAST (Sampson and Albergo, 1984), ELISAs (Burks, et al. 1986) immunoblotting (Burks, et al. 1988), and *in vivo* skin tests (Sampson and Albergo 1984). Objective clinical symptoms can be monitored before and after the administration of the modified allergen to determine any change in the clinical symptoms.

It may be of value to identify IgEs which interact with conformational rather than linear epitopes. Due to the complexity and heterogeneity of patient serum, it may be difficult to employ a standard immobilized allergen affinity-based approach to directly isolate these IgEs in quantities sufficient to permit their characterization. These problems can be avoided by isolating some or all of the IgEs which interact with conformational epitopes from a combinatorial IgE phage display library.

Steinberger et al. (Steinberger, P., Kraft D. and Valenta R. (1996) "Construction of a combinatorial IgE library from an allergic patient: Isolation and characterization of human IgE Fabs with specificity for the major Timothy Grass pollen antigen," *Phl p. 5 J. Biol. Chem.* 271, 10967-10972) prepared a combinatorial IgE phage display library from mRNA

isolated from the peripheral blood mononuclear cells of a grass allergic patient. Allergen-specific IgEs were selected by panning filamentous phage expressing IgE Fabs on their surfaces against allergen immobilized on the wells of 96 well microtiter plates. The cDNAs were then isolated from allergen-binding phage and transformed into E coli for the production of large quantities of monoclonal, recombinant, allergen-specific IgE Fabs.

If native allergen or full length recombinant allergen is used in the panning step to isolate phage, then Fabs corresponding to IgEs specific for conformational epitopes should be included among the allergen-specific clones identified. By screening the individual recombinant IgE Fabs against denatured antigen or against the relevant linear epitopes identified for a given antigen, the subset of conformation-specific clones which do not bind to linear epitopes can be defined.

To determine whether the library screening has yielded a complete inventory of the allergen-specific IgEs present in patient serum, an immunocompetition assay can be performed. Pooled recombinant Fabs would be preincubated with immobilized allergen. After washing to remove unbound Fab, the immobilized allergen would then be incubated with patient serum. After washing to remove unbound serum proteins, an incubation with a reporter-coupled secondary antibody specific for IgE Fc domain would be performed. Detection of bound reporter would allow quantitation of the extent to which serum IgE was prevented from binding to allergen by recombinant Fab. Maximal, uncompetited serum IgE binding would be determined using allergen which had not been preincubated with Fab or had been incubated with nonsense Fab. If IgE binding persists in the face of competition from the complete set of allergen-specific IgE Fab clones, this experiment can be repeated using denatured antigen to determine whether the epitopes not represented among the cloned Fabs are linear or conformational.

Production of Recombinant or Modified Allergens

A modified allergen will typically be made using recombinant techniques. Expression in a procaryotic or eucaryotic host including bacteria, yeast, and baculovirus-insect cell systems are typically used to produce large (mg) quantities of the modified allergen. It is also possible to make the allergen synthetically, if the allergen is not too large, for example, less than about 25-40 amino acids in length.

Production of Transgenic Plants and Animals

Transgenic plants or animals expressing the modified allergens have two purposes. First, they can be used as a source of modified allergen for use in immunotherapy and second, appropriately modified plants or animals can be substituted for the original plant or animal, making immunotherapy unnecessary. Furthermore, it is possible that eating modified peanuts or cod fish, for example, could have either or both of two effects: (1) not imparting an allergic response on their own and (2) conferring protection from the unmodified source by acting as an immunotherapeutic agent for the unmodified source. Methods for engineering of plants and animals are well known and have been for a decade. For example, for plants see Day, (1996) *Crit. Rev. Food Sci. & Nut.* 36(S), 549-567, the teachings of which are incorporated herein. See also Fuchs and Astwood (1996) *Food Tech.* 83-88. Methods for making recombinant animals are also well established. See, for example, Colman, A" Production of therapeutic proteins in the milk of transgenic livestock" (1998) *Biochem. Soc. Symp.* 63, 141-147; Espanion and Niemann, (1996) *DTW Dtxch Tierarztl Wochenschr* 103(8-9), 320-328; and Colman, *Am. J. Clin. Nutr.* 63(4), 639S-645S, the teachings of which are incorporated herein. One can also induce site specific changes using homologous recombination and/or triplex forming oligomers. See, for example, Rooney and Moore, (1995) *Proc. Natl. Acad. Sci. USA* 92, 2141-2149; Agrawal, et al., *BioWorld Today*, vol. 9, no. 41, p. 1 "Chimeriplasty -

Gene Surgery, Not Gene Therapy - Fixes Flawed Genomic Sequences" David N. Leff.

Production and Screening of Compounds blocking IgE Binding Sites

Once the IgE binding sites have been identified, it is also possible to block or limit binding to one or more of these sites by reacting the allergen with a compound that does not prevent the allergen from activating T cells, and in some embodiments does not significantly alter or decrease IgG binding capacity, resulting in a modified allergen similar in functionality to that produced by mutation. There are two principal ways to obtain compounds which block IgE binding sites: combinatorial libraries and combinatorial chemistry.

Identification of Compounds That Mask IgE Binding Sites through Application of Combinatorial Chemistry

In some cases it may be preferable to utilize non-peptide compounds to block binding of IgE to the allergen by masking the IgE binding epitope. This can be accomplished by using molecules that are selected from a complex mixture of random molecules in what has been referred to as "*in vitro* genetics" or combinatorial chemistry (Szostak, *TIBS* 19:89, 1992). In this approach a large pool of random and defined sequences is synthesized and then subjected to a selection and enrichment process. The selection and enrichment process involves the binding of the IgE binding epitopes to a solid support, followed by interaction with the products of various combinatorial libraries. Those molecules which do not bind these molecules at all are removed immediately by elution with a suitable solvent. Those molecules which bind to the epitopes will remain bound to the solid support, whereas, unbound compounds will be removed from the column. Those compounds bound to the column can be removed, for example, by competitive binding. Following removal of these compounds, the compounds which have bound can be identified, using methodology well known to those of skill in the art,

to isolate and characterize those compounds which bind to or interact with IgE binding epitopes. The relative binding affinities of these compounds can be compared and optimum compounds identified using competitive binding studies which are well known to those of skill in the art.

5 Identification of Compounds That Interact with IgE Binding Sites
through Application of Combinatorial Phage Display Libraries

Recombinant, monoclonal Fabs directed against conformational epitopes, identified as described above, can be used as reagents to assist in the definition of the biochemical nature of these epitopes. Cross-linking studies employing derivatized Fabs can be employed to label amino acid residues in the vicinity of the epitopes. Similarly, the Fabs can be used in protease protection studies to identify those domains of the allergen protein which are shielded from protease degradation by pre-binding of a specific Fab. Experiments employing recombinant monoclonal Fabs as reagents to label or protect from labeling should permit at least partial elucidation of the structures of conformational epitopes.

"Humanized" recombinant Fabs should bind to allergens if injected into a patient and thus prevent the binding of these allergens to native IgE. Since the Fabs cannot interact with the Fcε receptor, the binding of the IgE Fabs to allergen would not be expected to elicit mast cell degranulation. Allergen should be neutralized as it is by protective IgGs.

Anti-idiotypic antibodies directed against the conformational epitope-specific Fabs should resemble the conformation epitopes themselves. Injection of these anti-idiotypic antibodies should induce the production of anti-anti-idiotypic IgGs which would recognize, bind to and inactivate the conformational epitopes. The method through which the anti-idiotypic antibodies would be produced (i.e. animal immunization, "*in vitro*" immunization or recombinant phage display library) would have to be determined. Similarly, the possibility that the anti-idiotypic antibodies (which

resemble the conformational epitopes) would be recognized by patient IgEs and induce mast cell degranulation needs to be considered.

II. Diagnostic and Therapeutic Procedures Using Modified Allergens.

It is important to administer the modified allergen to an individual (human or animal) to decrease the clinical symptoms of allergic disease by using a method, dosage, and carrier which are effective. Allergen will typically be administered in an appropriate carrier, such as saline or a phosphate saline buffer. Allergen can be administered by injection subcutaneously, intramuscularly, or intraperitoneally (most humans would be treated by subcutaneous injection), by aerosol, inhaled powder, or by ingestion.

Therapy or desensitization with the modified allergens can be used in combination with other therapies, such as allergen-non-specific anti-IgE antibodies to deplete the patient of allergen-specific IgE antibodies (Boulet, et al. (1997) 155:1835-1840; Fahy, et al. (1997) *American J Respir. Crit. Care Med.* 155:1828-1834; Demoly, P. and Bousquet (1997) *J Am J Resp. Crit. Care Med.* 155:1825-1827), or by the pan specific anti-allergy therapy described in U. S. Serial No. 08/090,375 filed June 4, 1998, by M. Caplan and H. Sosin. Therapy with the modified allergen can also be administered in combination with an adjuvant such as IL 12, IL 16, IL 18, Ifn- γ .

The nucleotide molecule encoding the modified allergen can also be administered directly to the patient, for example, in a suitable expression vector such as a plasmid, which is injected directly into the muscle or dermis, or through administration of genetically engineered cells.

In general, effective dosages will be in the picogram to milligram range, more typically microgram to milligram. Treatment will typically be between twice/weekly and once a month, continuing for up to three to five years, although this is highly dependent on the individual patient response.

The modified allergen can also be used as a diagnostic to characterize

the patient's allergies, using techniques such as those described in the examples.

EXAMPLES

Peanut allergy is one of the most common and serious of the immediate hypersensitivity reactions to foods in terms of persistence and severity of reaction. Unlike the clinical symptoms of many other food allergies, the reactions to peanuts are rarely outgrown, therefore, most diagnosed children will have the disease for a lifetime (Sampson, H.A., and Burks, A.W. (1996) *Annu. Rev. Nutr.* 16, 161-77; Bock, S.A. (1985) *J. Pediatr.* 107, 676-680). The majority of cases of fatal food-induced anaphylaxis involve ingestion of peanuts (Sampson et al., (1992) *NEJM* 327, 380-384; Kaminogawa, S. (1996) *Biosci. Biotech. Biochem.* 60, 1749-1756). The only effective therapeutic option currently available for the prevention of a peanut hypersensitivity reaction is food avoidance. Unfortunately, for a ubiquitous food such as a peanut, the possibility of an inadvertent ingestion is great.

The examples described below demonstrate identification, modification, and assessment of allergenicity of the major peanut allergens, Ara h 1, Ara h 2, and Ara h 3. Detailed experimental procedures are included for Example 1. These same procedures were used for Examples 2-5. The nucleotide sequences of Ara h 1, Ara h 2, and Ara h 3, are shown in SEQ ID NOs. 1, 3, and 5, respectively. The amino acid sequences of Ara h 1, Ara h 2, and Ara h 3 are shown in SEQ ID NOs. 2, 4, and 6 respectively.

Example 1: Identification of linear IgE binding epitopes.

Due to the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, there is increasing interest in defining the allergenic proteins and exploring ways to decrease the risk to the peanut-sensitive individual. Various studies over the last several years

have identified the major allergens in peanuts as belonging to different families of seed storage proteins (Burks, et al. (1997) *Eur. J. Biochem.* 245, 334-339; Stanley, et al. (1997) *Arch. Biochem. Biophys.* 342, 244-253). The major peanut allergens Ara h 1, Ara h 2, and Ara h 3 belong to the vicilin, conglutin and glycinin families of seed storage proteins, respectively. These allergens are abundant proteins found in peanuts and are recognized by serum IgE from greater than 95% of peanut sensitive individuals, indicating that they are the major allergens involved in the clinical etiology of this disease (Burks, et al. (1995) *J. Clinical Invest.*, 96, 1715-1721). The genes encoding Ara h 1 (SEQ ID NO. 1), Ara h 2 (SEQ ID NO. 3), and Ara h 3 (SEQ ID NO. 5) and the proteins encoded by these genes (SEQ ID NO. 2, 4, 6) have been isolated and characterized. The following studies were conducted to identify the IgE epitopes of these allergens recognized by a population of peanut hypersensitive patients and a means for modifying their affinity for IgE.

Experimental Procedures

Serum IgE. Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 yrs) was used to determine relative binding affinities between wild type and mutant Ara h 1 synthesized epitopes. The patients had either a positive double-blind, placebo-controlled, food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension; Burks, et al. (1988) *J. Pediatr.* 113, 447-451). At least 5 ml of venous blood was drawn from each patient, allowed to clot, and serum was collected. A serum pool from 12 to 15 patients was made by mixing equal aliquots of serum IgE from each patient. The pools were then used in immunoblot analysis.

Peptide synthesis. Individual peptides were synthesized on a derivatized cellulose membrane using 9-fluorenylmethoxycarbonyl (Fmoc) amino acid active esters according to the manufacturer's instructions

(Genosys Biotechnologies, Woodlands, Texas; Fields, G.B. and Noble, R.L. (1990) *Int. J. Peptide Protein Res.* 35, 161-214). Fmoc-amino acids (N-terminal blocked) with protected side chains were coupled in the presence of 1-methyl-2-pyrrolidone to a derivatized cellulose membrane. Following washing with dimethylformamide (DMF), unreacted terminal amino groups were blocked from further reactions by acetylation with acetic anhydride. The N-terminal Fmoc blocking group was then removed by reaction with 20% piperidine and 80% DMF, v/v. The membrane was washed in DMF followed by methanol, the next reactive Fmoc-amino acid was then coupled as before, and the sequence of reactions was repeated with the next amino acid. When peptide synthesis was complete, the side chains were deprotected with a mixture of dichloromethane (DCM), trifluoroacetic acid, and triisobutylsilane (1.0:1.0:0.5), followed by successive washes in DCM, DMF, and methanol. Peptides synthesis reactions were monitored by bromophenol blue color reactions during certain steps of synthesis. Cellulose derivitised membranes and Fmoc-amino acids were supplied by Genosys Biotechnologies. All other chemical were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) or Fluka (Buchs, Switzerland). Membranes were either probed immediately or stored at -20°C until needed.

IgE binding assays. Cellulose membranes containing synthesized peptides were washed 3 times in Tris-buffered saline (TBS; 136 mM NaCl, 2.7 mM KCl, and 50 mM trizma base pH 8.0) for 10 min at room temperature (RT) and then incubated overnight in blocking buffer: [TBS, 0.05% TweenTM 20; concentrated membrane blocking buffer supplied by Genosys; and sucrose (0.0:1.0:0.5)]. The membrane was then incubated in pooled sera diluted in 1:5 in 20 mM Tris-Cl pH7.5, 150 mM NaCl, and 1% bovine serum albumin overnight at 4°C. Primary antibody was detected with ¹²⁵I-labeled equine anti-human IgE (Kallestad, Chaska, MN).

Quantitation of IgE binding. Relative amounts of IgE binding to individual peptides were determined by a Bio-Rad (Hercules, CA) model GS-700 imaging laser densitometer and quantitated with Bio-Rad molecular analyst software. A background area was scanned and subtracted from the obtained values. Following quantitation, wild type intensities were normalized to a value of one and the mutants were calculated as percentages relative to the wild type.

Synthesis and purification of recombinant Ara h 2 protein. cDNA encoding Ara h 2 was placed in the pET-24b expression vector. The pET-24 expression vector places a 6 x histidine tag at the carboxyl end of the inserted protein. The histidine tag allows the recombinant protein to be purified by affinity purification on a nickel column (HisBind resin). Recombinant Ara h 2 was expressed and purified according to the instructions of the pET system manual. Briefly, expression of the recombinant Ara h 2 was induced in 200 ml cultures of strain BL21(DE3) E. coli with 1 mM IPTG at mid log phase. Cultures were allowed to continue for an additional 3 hours at 36°C. Cells were harvested by centrifugation at 2000 x g for 15 minutes and then lysed in denaturing binding buffer (6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Lysates were cleared by centrifugation at 39,000 x g for 20 minutes followed by filtration through 0.45 micron filters. The cleared lysate was applied to a 10 ml column of HisBind resin, washed with imidazole wash buffer (20 mM imidazole, 6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The recombinant Ara h 2 was then released from the column using elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The elution buffer was replaced with phosphate buffered saline by dialysis. The purification of recombinant Ara h 2 was followed by SDS PAGE and immunoblots. Peanut specific serum IgE was used as a primary antibody.

Skin prick tests. The ability of purified native and recombinant Ara h 2 to elicit the IgE mediated degranulation of mast cells was evaluated using prick skin tests in a peanut allergic individual. An individual meeting the criteria for peanut allergy (convincing history or positive double blind placebo controlled food challenge) and a non-allergic control were selected for the testing. Purified native and recombinant Ara h 2 and whole peanut extract (Greer Laboratories, Lenoir, N.C.) were tested. Twenty microliters of the test solution were applied to the forearm of the volunteer and the skin beneath pricked with a sterile needle. Testing was started at the lowest concentration (less than or equal to 1 mg/ml) and increased ten fold each round to the highest concentration or until a positive reaction was observed. Mean diameters of the wheal and erythema were measured and compared to the negative saline control. A positive reaction was defined as a wheal 3mm larger than the negative control. Histamine was used as the positive control.

Results

Identification of the linear IgE-binding epitopes of Ara h 1, Ara h 2 and Ara h 3 allergens. Epitope mapping was performed on the Ara h 1, Ara h 2 and Ara h 3 allergens by synthesizing each of these proteins in 15 amino acid long overlapping peptides that were offset from each other by 8 amino acids. The peptides were then probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. This analysis resulted in multiple IgE binding regions being identified for each allergen. The exact position of each IgE binding epitope was then determined by re-synthesizing these IgE reactive regions as 10 or 15 amino acid long peptides that were offset from each other by two amino acids. These peptides were probed with the same pool of serum IgE from peanut sensitive patients as used before. An example of this procedure for each of the peanut allergens is shown in Fig. 1-3 (Ara h 1 - Fig. 1; Ara h 2 - Fig. 2; Ara h 3 - Fig. 3). This analysis

revealed that there were 23 linear IgE binding epitopes on Ara h 1, 10 epitopes on Ara h 2, and 4 epitopes on Ara h 3.

In an effort to determine which, if any, of the epitopes were recognized by the majority of patients with peanut hypersensitivity, each set of epitopes identified for the peanut allergens were synthesized and then probed individually with serum IgE from 10 different patients. All of the patient sera tested recognized multiple epitopes.

Table 1 shows the amino acid sequence and position of each epitope within the Ara h 1 protein of all 23 IgE binding epitopes mapped to this molecule. Table 2 shows the amino acid sequence and position of each epitope within the Ara h 2 protein of all 10 IgE binding epitopes mapped to this molecule. Table 3 shows the amino acid sequence and position of each epitope within the Ara h 3 protein of all 4 IgE binding epitopes mapped to this molecule.

Four epitopes of the Ara h 1 allergen (peptides 1,3,4,17 of Table 1), three epitopes of the Ara h 2 allergen (peptides 3,6,7 of Table 2), and 1 epitope of the Ara h 3 allergen (peptide 2 of Table 3) were immunodominant.

Table 1. Ara h I IgE Binding Epitopes

EPITOPE	AA SEQUENCE	POSITION
1	AKSSPYOKKT	25-34
2	<u>QEPDDLKQKA</u>	48-57
3	<u>LEYDPRLVYD</u>	65-74
4	<u>GERTRGROPG</u>	89-98
5	<u>PGDYDDDDRQ</u>	97-106
6	<u>PRREEGGRWG</u>	107-116
7	<u>REREEDWRQP</u>	123-132
8	<u>EDWRRPSHQQ</u>	134-143
9	<u>QPRKIRPEGR</u>	143-152
10	<u>TPGQFEDFFP</u>	294-303
11	<u>SYLQEFSRNT</u>	311-320
12	<u>FNAEFNEIRR</u>	325-334
13	<u>EQEERGQRRW</u>	344-353
14	<u>DITNPINLRE</u>	393-402
15	<u>NNFGKLFEVK</u>	409-418
16	<u>GTGNLELVAV</u>	461-470
17	<u>RRYTARLKEG</u>	498-507
18	<u>ELHLLGFGIN</u>	525-534
19	<u>HRIFLAGDKD</u>	539-548
20	<u>IDQIEKQAKD</u>	551-560
21	<u>KDLAFPGSGE</u>	559-568
22	<u>KESHFVSARP</u>	578-587
23	<u>PEKESPEKED</u>	597-606

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 2.

Table 2. Ara h 2 IgE Binding Epitopes

EPITOPE	AA SEQUENCE	POSITION
1	<u>HASARQOWEL</u>	15-24
2	<u>QWELQGDRRC</u>	21-30
3	<u>DRRCQSOLER</u>	27-36
4	<u>LRPCEQHLMQ</u>	39-48
5	<u>KIQRDEDSYE</u>	49-58
6	<u>YERDPYSPSQ</u>	57-66
7	<u>SQDPYSPSPY</u>	65-74
8	<u>DRLOGRQQEQ</u>	115-124
9	<u>KRELRLNPQQ</u>	127-136
10	<u>QRCDLDVESG</u>	143-152

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 4.

Table 3. Ara h 3 IgE Binding Epitopes

EPITOPE	AA SEQUENCE	POSITION
1	<u>IETWNPNNQEFECAG</u>	33-47
2	<u>GNIFSGFTPEFLEQA</u>	240-254
3	<u>VTVRGGLRILSPDRK</u>	279-293
4	<u>DEDEYDYDEEDRRRG</u>	303-317

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 6.

Example 2 : Modification of peanut allergens to decrease allergenicity.

The major linear IgE binding epitopes of the peanut allergens were mapped using overlapping peptides synthesized on an activated cellulose membrane and pooled serum IgE from 15 peanut sensitive patients, as described in Example 1. The size of the epitopes ranged from six to fifteen amino acids in length. The amino acids essential to IgE binding in each of the epitopes were determined by synthesizing duplicate peptides with single amino acid changes at each position. These peptides were then probed with pooled serum IgE from 15 patients with peanut hypersensitivity to determine if the changes affected peanut-specific IgE binding. For example, epitope 9 in Table 1 was synthesized with an alanine or methionine residue substituted for one of the amino acids and probed. The following amino acids were substituted (first letter is the one-letter amino acid code for the residue normally at the position, the residue number, followed by the amino acid that was substituted for this residue; the numbers indicate the position of each residue in the Ara h 1 protein, SEQ ID NO. 2): Q143A, P144A; R145A; K146A; I147A; R148A; P149A; E150A; G151A; R152A; Q143M; P144M; R145M; K146M; I147M; R148M; P149M; E150M; G151M; R152M. The immunoblot strip containing the wild-type and mutated peptides of epitope 9 showed that binding of pooled serum IgE to individual peptides was dramatically reduced when either alanine or methionine was substituted for each of the amino acids at positions 144, 145, and 147-150 of Ara h 1 shown in SEQ ID NO. 2. Changes at positions 144, 145, 147, and 148 of Ara h 1 shown in SEQ ID NO. 2 had the most dramatic effect when methionine was substituted for the wild-type amino acid, resulting in less than 1% of peanut specific IgE binding to these peptides. In contrast, the substitution of an alanine for arginine at position 152 of Ara h 1 shown in SEQ ID NO. 2 resulted in increased IgE binding. The remaining Ara h 1 epitopes, and the Ara h 2 and Ara h 3 epitopes, were tested in the same manner and the

intensity of IgE binding to each spot was determined as a percentage of IgE binding to the wild-type peptide. Any amino acid substitution that resulted in less than 1% of IgE binding when compared to the wild type peptide was noted and is indicated in Tables 4-6. Table 4 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 1 epitopes. Table 5 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 2 epitopes. Table 6 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 3 epitopes. This analysis indicated that each epitope could be mutated to a non-IgE binding-peptide by the substitution of a single amino acid residue.

The results discussed above for Ara h 1, Ara h 2, and Ara h 3 demonstrate that once an IgE binding site has been identified, it is possible to reduce IgE binding to this site by altering a single amino acid of the epitope. The observation that alteration of a single amino acid leads to the loss of IgE binding in a population of peanut-sensitive individuals is significant because it suggests that while each patient may display a polyclonal IgE reaction to a particular allergen, IgE from different patients that recognize the same epitope must interact with that epitope in a similar fashion. Besides finding that many epitopes contained more than one residue critical for IgE binding, it was also determined that more than one residue type (ala or met) could be substituted at certain positions in an epitope with similar results. This allows for the design of a hypoallergenic protein that would be effective at blunting allergic reactions for a population of peanut sensitive individuals. Furthermore, the creation of a plant producing a peanut where the IgE binding epitopes of the major allergens have been removed should prevent the development of peanut hypersensitivity in individuals genetically predisposed to this food allergy.

Table 4: Amino Acids Critical to IgE Binding of Ara h 1

EPITOPE	AA SEQUENCE	POSITION
1	AKSS <u>P</u> YQ <u>K</u> KT	25-34
2	QEP <u>D</u> DLKQKA	48-57
3	LEY <u>D</u> PRLVYD	65-74
4	GER <u>T</u> R <u>G</u> RQPG	89-98
5	PGDYDD <u>D</u> RRQ	97-106
6	PRREE <u>G</u> GRWG	107-116
7	REREEDWR <u>Q</u> P	123-132
8	EDW <u>R</u> RPSHQQ	134-143
9	QPRKIRPEGR	143-152
10	TPGQFED <u>F</u> FP	294-303
11	SYLQEF <u>S</u> RNT	311-320
12	FNAEFNEIRR	325-334
13	EQEERGQRRW	344-353
14	DITNP <u>I</u> N <u>L</u> RE	393-402
15	NNFGKL <u>F</u> EVK	409-418
17	<u>R</u> RYTARLKEG	498-507
18	EL <u>H</u> LLGFGIN	525-534
19	HRIFLAGD <u>K</u> D	539-548
20	IDQIEKQ <u>A</u> K <u>D</u>	551-560
21	KDLA <u>F</u> PGSGE	559-568
22	KESHFVS <u>A</u> RP	578-587

Note. The Ara h 1 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 1 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by a single patient who was no longer available to the study. All of these sequences can be found in SEQ ID NO 2.

Table 5. Amino Acids Critical to IgE Binding of Ara h 2

EPITOPE	AA SEQUENCE	POSITION
1	HASAR Q <u>Q</u> WEL	15-24
2	<u>Q</u> WEL <u>Q</u> GDRRC	21-30
3	D <u>R</u> R <u>C</u> Q <u>S</u> Q <u>L</u> E <u>R</u>	27-36
4	<u>L</u> R <u>P</u> C <u>E</u> <u>Q</u> H <u>L</u> M <u>Q</u>	39-48
5	<u>K</u> I <u>Q</u> <u>R</u> <u>D</u> E <u>D</u> S <u>Y</u> E	49-58
6	YER <u>D</u> P <u>Y</u> S <u>P</u> S <u>Q</u>	57-66
7	<u>S</u> Q <u>D</u> P <u>Y</u> S <u>P</u> S <u>P</u> Y	65-74
8	DR <u>L</u> <u>Q</u> <u>G</u> <u>R</u> Q <u>Q</u> E <u>Q</u>	115-124
9	<u>K</u> <u>R</u> E <u>L</u> <u>R</u> N <u>L</u> P <u>Q</u> Q	127-136
10	Q <u>R</u> C <u>D</u> <u>L</u> D <u>V</u> E <u>S</u> G	143-152

Note. The Ara h 2 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 2 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. All of these sequences can be found in SEQ ID NO 4.

Table 6. Amino Acids Critical to IgE-Binding of Ara h 3.

EPITOPE	AA SEQUENCE	POSITION
1	IETWNP <u>P</u> NNQEFECAG	33-47
2	GN <u>I</u> FS <u>G</u> FT <u>P</u> EF <u>L</u> EQA	240-254
3	VTVRG <u>G</u> <u>L</u> R <u>I</u> L <u>S</u> P <u>D</u> RK	279-293
4	DEDEY <u>E</u> <u>Y</u> <u>D</u> E <u>D</u> <u>R</u> <u>R</u> RG	303-317

Note. The Ara h 3 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 3 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined. All of these sequences can be found in SEQ ID NO 6.

Example 3: A Modified Ara h 2 Protein Binds less IgE But Similar Amounts of IgG.

In order to determine the effect of changes to multiple epitopes within the context of the intact allergen, four epitopes (including the three immunodominant epitopes) of the Ara h 2 allergen were mutagenized and the protein produced recombinantly. The amino acids at position 20, 31, 60, and 67 of the Ara h 2 protein (shown in SEQ ID NO. 4 and Figure 4A) were changed to alanine by mutagenizing the gene encoding this protein by standard techniques. These residues are located in epitopes 1, 3, 6, and 7 and represent amino acids critical to IgE binding that were determined in Example 2. The modified and wild-type versions of this protein were produced and immunoblot analysis performed using serum from peanut sensitive patients. These results showed that the modified version of this allergen bound significantly less IgE than the wild type version of these recombinant proteins (Figure 4B) but bound similar amounts of IgG.

Example 4: A modified Ara h 2 protein retains the ability to stimulate T-cells to proliferate.

The modified recombinant Ara h 2 protein described in Example 3 was used in T-cell proliferation assays to determine if it retained the ability to activate T cells from peanut sensitive individuals. Proliferation assays were performed on T-cell lines grown in short-term culture developed from six peanut sensitive patients. T-cells lines were stimulated with either 50 μ g of crude peanut extract, 10 μ g of native Ara h 2, 10 μ g of recombinant wild-type Ara h2, or 10 μ g of modified recombinant Ara h 2 protein and the amount of 3H-thymidine determined for each cell line. Results were expressed as the average stimulation index (SI) which reflected the fold increase in 3H-thymidine incorporation exhibited by cells challenged with allergen when compared with media treated controls (Figure 5).

Example 5: A Modified Ara h 2 Protein Elicits a Smaller Wheal and Flare in Skin Prick Tests of a Peanut Sensitive Individual.

5 The modified recombinant Ara h 2 protein described in Example 3 and the wild type version of this recombinant protein were used in a skin prick test of a peanut sensitive individual. Ten micrograms of these proteins were applied separately to the forearm of a peanut sensitive individual, the skin pricked with a sterile needle, and 10 minutes later any wheal and flare that developed was measured. The wheal and flare produced by the wild-type Ara h 2 protein (8 mm X 7 mm) was approximately twice as large as that produced by the modified Ara h 2 protein (4 mm X 3mm). A control subject (no peanut hypersensitivity) tested with the same proteins had no visible wheal and flare but, as expected, gave positive results when challenged with histamine. In addition, the test subject gave no positive results when tested with PBS alone. These results indicate that an allergen with only 40% of its IgE binding epitopes modified (4/10) can give measurable reduction in reactivity in an *in vivo* test of a peanut sensitive patient.

15 These same techniques can be used with the other known peanut allergens, Ara h 1 (SEQ ID NO 1 and 2), Ara h 3 (SEQ ID NO. 5 and 6), or any other allergen.

20 Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the appended claims.

We claim:

1. A method of making a modified allergen which is less reactive with IgE comprising
 - (a) identifying IgE binding sites in an allergen;
 - (b) modifying the allergen by mutating at least one amino acid in an IgE binding site or reacting the allergen with a compound blocking binding to at least one amino acid in an IgE binding site;
 - (c) screening for IgE binding to the modified allergen using serum or antibodies from a pooled patient population and screening for activation of T cells; and
 - (d) selecting the modified allergens which have decreased binding to IgE as compared to the unmodified allergen and which activate T cells.
2. The method of claim 1 further comprising screening for binding of the modified allergen for binding to IgG and selecting the modified allergens which have decreased binding to IgE, activate T cells and bind to IgG.
3. The method of claim 1 wherein the modified allergen is mutated in the center of one or more of the IgE binding sites.
4. The method of claim 1 wherein the modified allergen is mutated by substituting a hydrophobic amino acid in the center of one or more of the IgE binding sites with a neutral or hydrophilic amino acid.
5. The method of claim 1 wherein binding of IgE to the modified allergen is blocked by reaction of a compound with at least one amino acid present in an IgE binding site.
6. The method of claim 5 wherein binding of IgE is blocked by reaction of the allergen with an antibody which blocks binding to one or more IgE sites but allows the allergen to still activate T cells.
7. The method of claim 1 wherein the modified allergen is a portion of a protein.

8. The method of claim 1 wherein the modified allergen is formulated with an adjuvant selected from the group consisting of IL 12, IL 16, IL 18, Ifn- γ or immune stimulatory sequences.
9. The method of claim 1 wherein the modified allergen is screened for initiation of a T cell helper 1 response.
10. The method of claim 1 wherein the modified allergen is made in a recombinant host selected from the group consisting of plants, animals, bacteria, yeast, fungi, and insect cells.
11. The method of claim 1 wherein the modified allergen is made in cells using site specific mutation.
12. The method of claim 1 wherein the modified allergen is made from a peanut allergen selected from the group consisting of Ara h 1, Ara h 2, and Ara h 3.
13. The method of claim 1 wherein the modified allergen is based on a protein obtained from a source selected from the group consisting of legumes, milks, grains, eggs, fish, crustaceans, mollusks, insects, molds, dust, grasses, trees, weeds, mammals, birds, and natural latexes.
14. A modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells.
15. The modified allergen of claim 14 wherein the modified allergen binds IgG.
16. The modified allergen of claim 14 made by the process of
 - (a) identifying one or more IgE binding sites in an allergen;
 - (b) mutating at least one amino acid in an IgE binding site;
 - (c) screening for IgE binding to the mutated allergen and activation of T cells by the mutated allergen; and

(d) selecting the modified allergens with decreased binding to IgE which activate T cells.

17. The modified allergen of claim 14 wherein the modified allergen is mutated in the center of one or more of the IgE binding sites.

18. The modified allergen of claim 14 wherein the modified allergen is mutated by substituting a hydrophobic amino acid in the center of one or more of the IgE binding sites with a neutral or hydrophilic amino acid.

19. The modified allergen of claim 14 wherein binding of IgE is blocked by reaction of a compound with at least one amino acid present in an IgE binding site.

20. The modified allergen of claim 19 wherein binding of IgE is blocked by reaction of the allergen with an antibody which blocks binding to one or more IgE sites but allows the allergen to still activate T cells.

21. The modified allergen of Claim 20 wherein the modified allergen does not have significantly altered or decreased IgG binding capacity.

22. The modified allergen of claim 14 which initiates a T cell helper 1 response.

23. The modified allergen of claim 14 wherein the allergen is a portion of a protein.

24. The modified allergen of claim 14 wherein the modified allergen is formulated with an adjuvant selected from the group consisting of IL 12, IL 16, IL 18, Ifn- γ and immune stimulatory sequences.

25. The modified allergen of claim 14 wherein the modified allergen is made in a transgenic plant or animal.

26. The modified allergen of claim 14 expressed in a recombinant host selected from the group consisting of plants and animals.

27. The modified allergen of claim 17 expressed in a recombinant host selected from the group consisting of bacteria, yeast, fungi, and insect cells.

28. The modified allergen of claim 14 wherein the modified allergen is based on a protein obtained from a source selected from the group consisting of legumes, milks, grains, eggs, fish, crustaceans, mollusks, insects, molds, dust, grasses, trees, weeds, mammals, birds, and natural latexes.
29. The modified allergen of claim 14 wherein the modified allergen is made from a peanut allergen selected from the group consisting of Ara h 1, Ara h 2, and Ara h 3.
30. A nucleotide molecule encoding a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 21 or 22.
31. The molecule of claim 30 in a vector for expression in a recombinant host.
32. A nucleotide molecule for causing a site specific mutation in a gene encoding a protein which yields a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.
33. A transgenic plant expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23.
34. A transgenic animal expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23.

35. A compound selectively binding to at least one amino acid in an IgE binding site of an allergen, wherein the site no longer binds IgE, but wherein the allergen is able to activate T cells, wherein the compound is obtained using a combinatorial library or combinatorial chemistry and screening for reaction with the allergen to produce bound allergen, followed by testing of the bound allergen for binding to IgE and activation of T cells.

36. A method to treat an individual to reduce the clinical response to an allergen comprising administering to the individual a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells, as defined by any of claims 14-29, in an amount and for a time sufficient to reduce the allergic reaction to the unmodified allergen.

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

Abstract

It has been determined that allergens, which are characterized by both humoral (IgE) and cellular (T cell) binding sites, can be modified to be less allergenic by modifying the IgE binding sites. The IgE binding sites can be converted to non-IgE binding sites by masking the site with a compound that prevents IgE binding or by altering as little as a single amino acid within the protein, most typically a hydrophobic residue towards the center of the IgE-binding epitope, to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, other than within the IgE-binding sites, while retaining the ability of the protein to activate T cells, and, in some embodiments by not significantly altering or decreasing IgG binding capacity. The examples use peanut allergens to demonstrate alteration of IgE binding sites. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding have been determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

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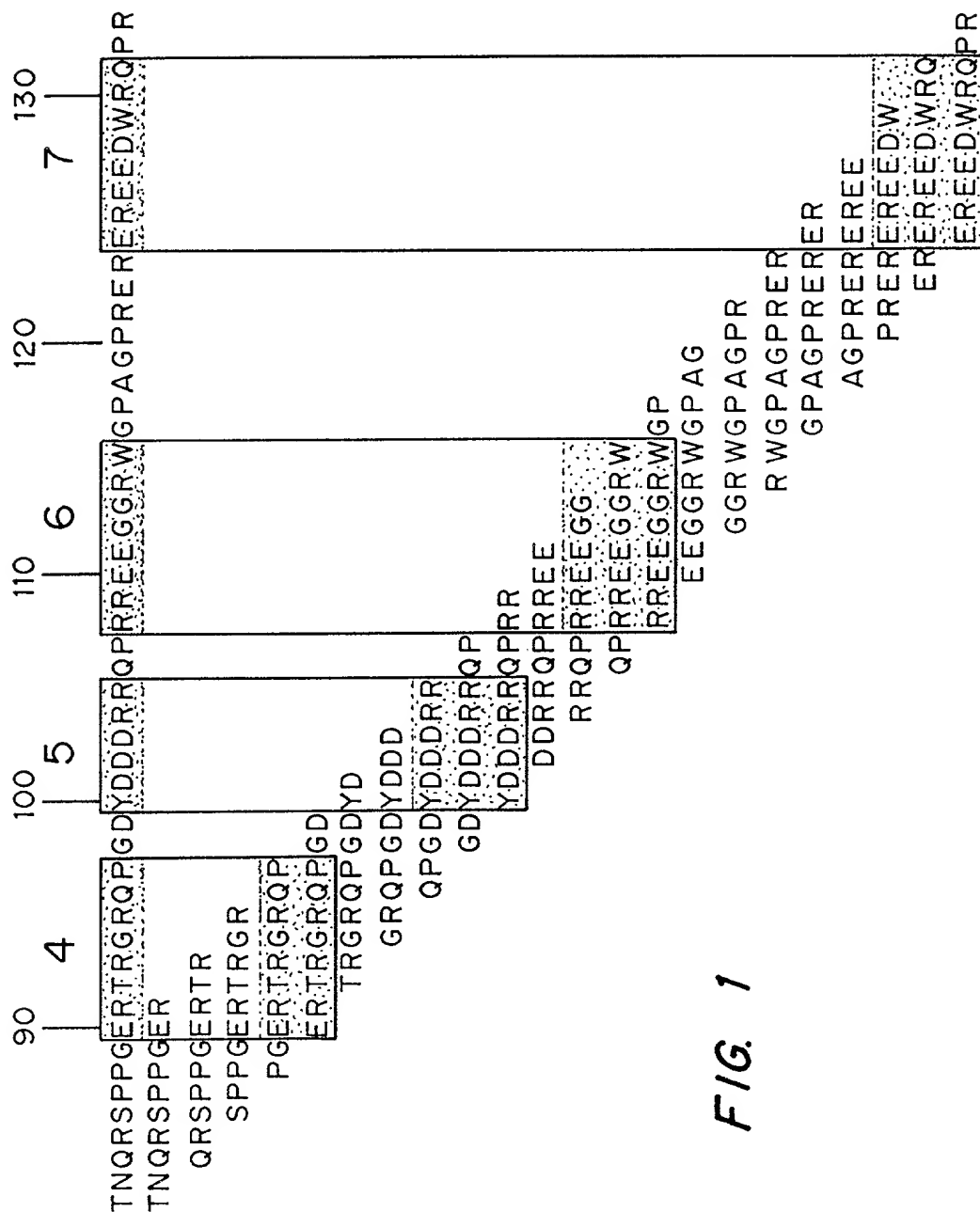


FIG. 1

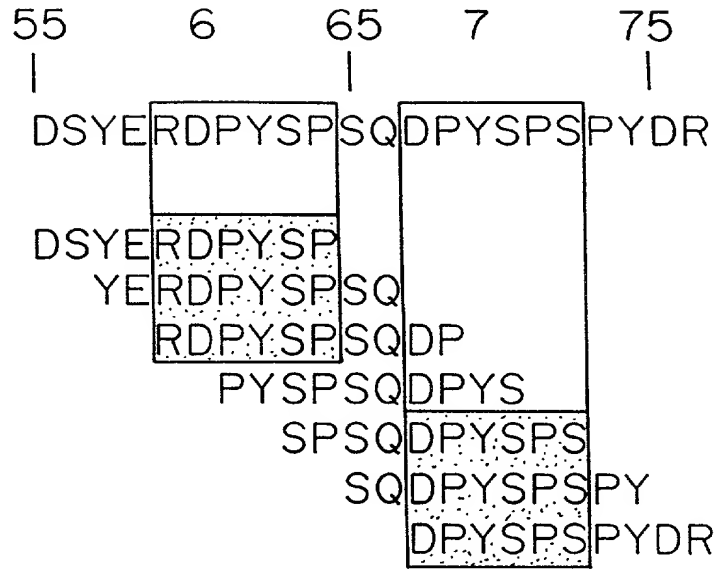


FIG. 2

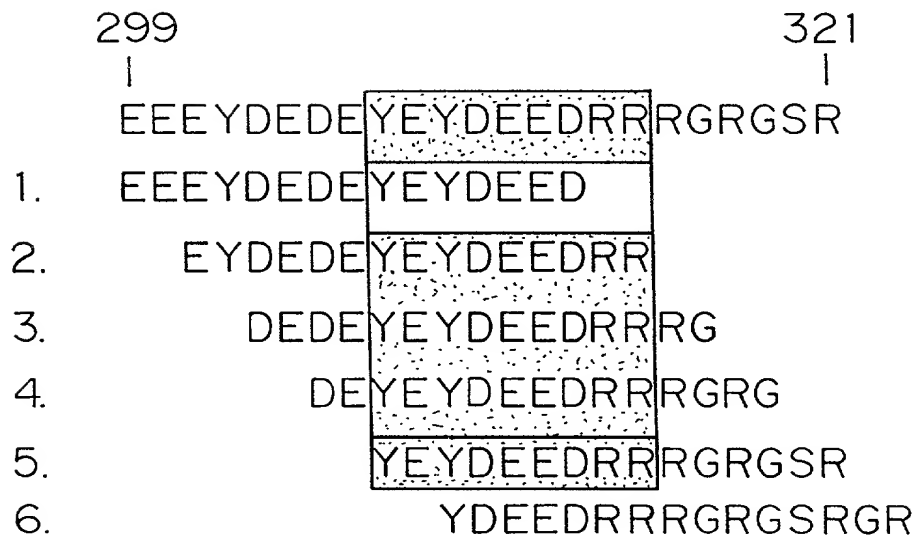


FIG. 3

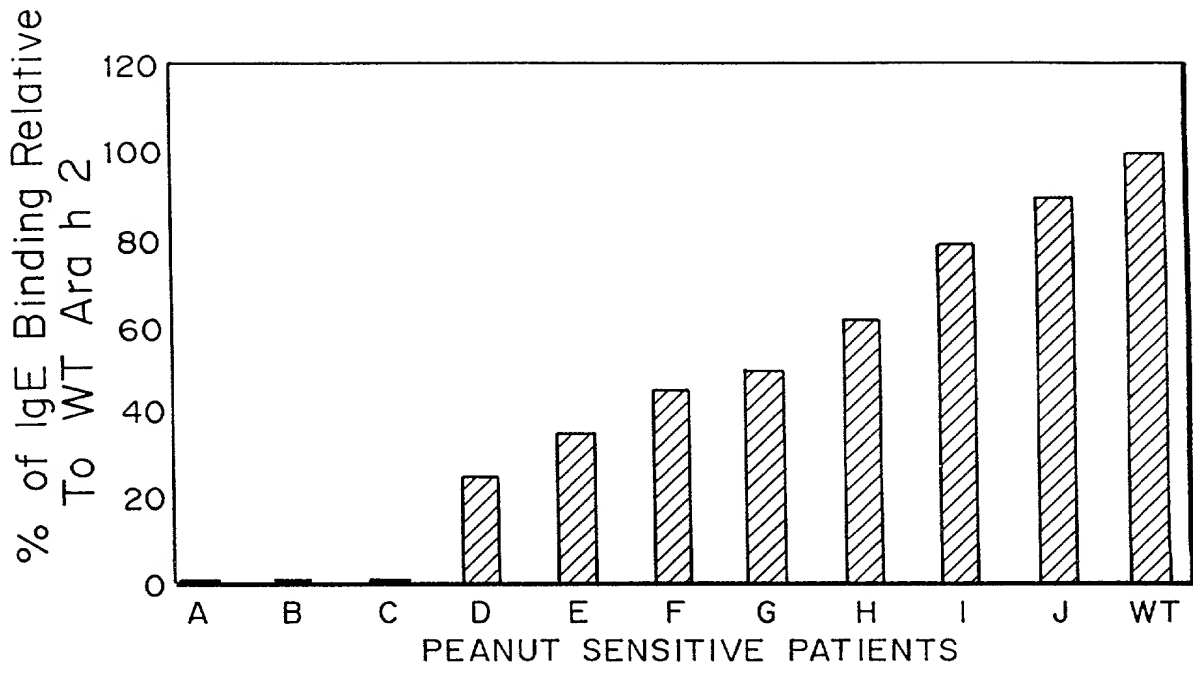


FIG. 4

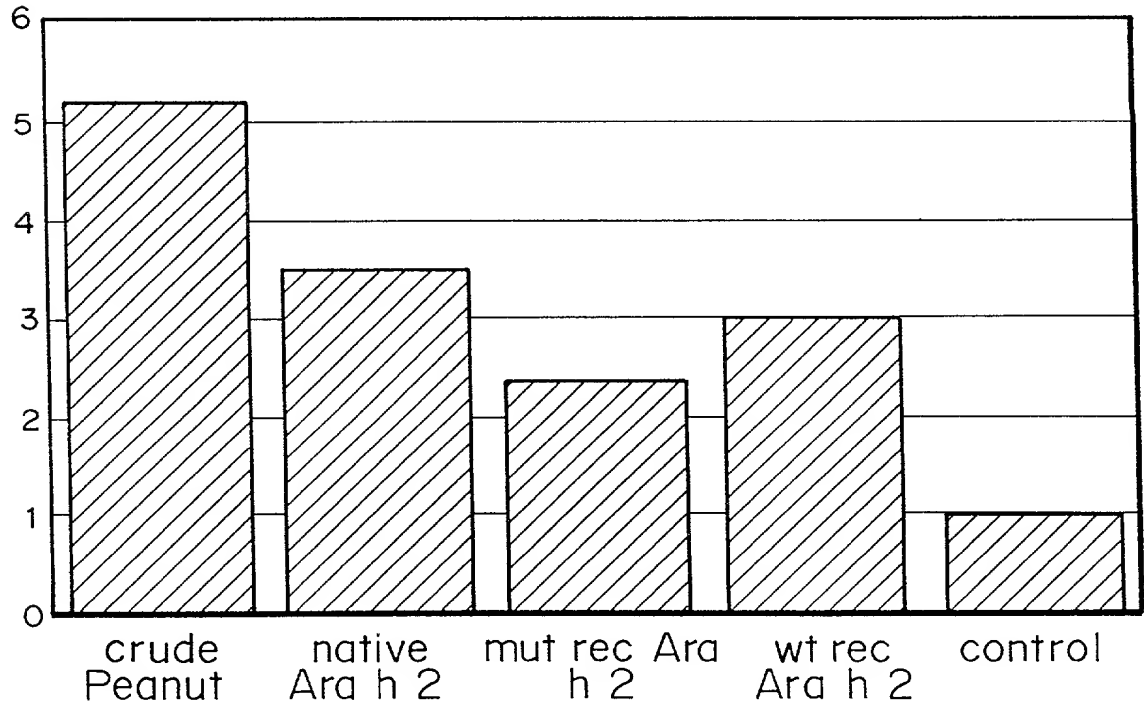


FIG. 5

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20

25

30

Gln Leu Glu Arg Ala Asn Leu Arg Pro Cys Glu Gln His Leu Met Gln

35

40

45

Lys Ile Gln Arg Asp Glu Asp Ser Tyr Glu Arg Asp Pro Tyr Ser Pro

50

55

60

Ser Gln Asp Pro Tyr Ser Pro Ser Pro Tyr Asp Arg Arg Gly Ala Gly

65

70

75

80

Ser Ser Gln His Gln Glu Arg Cys Cys Asn Glu Leu Asn Glu Phe Glu

85

90

95

Asn Asn Gln Arg Cys Met Cys Glu Ala Leu Gln Gln Ile Met Glu Asn

100

105

110

Gln Ser Asp Arg Leu Gln Gly Arg Gln Gln Glu Gln Gln Phe Lys Arg

115

120

125

Glu Leu Arg Asn Leu Pro Gln Gln Cys Gly Leu Arg Ala Pro Gln Arg

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20

25

30

Ile Glu Thr Trp Asn Pro Asn Asn Gln Glu Phe Glu Cys Ala Gly Val
 35 40 45

Ala Leu Ser Arg Leu Val Leu Arg Arg Asn Ala Leu Arg Arg Pro Phe
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Tyr Ser Asn Ala Pro Gln Glu Ile Phe Ile Gln Gln Gly Arg Gly Tyr
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Phe Gly Leu Ile Phe Pro Gly Cys Pro Arg His Tyr Glu Glu Pro His
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Thr Gln Gly Arg Arg Ser Gln Ser Gln Arg Pro Pro Arg Arg Leu Gln
 100 105 110

Gly Glu Asp Gln Ser Gln Gln Gln Arg Asp Ser His Gln Lys Val His
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Arg Phe Asp Glu Gly Asp Leu Ile Ala Val Pro Thr Gly Val Ala Phe
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Trp Leu Tyr Asn Asp His Asp Thr Asp Val Val Ala Val Ser Leu Thr
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Asp Thr Asn Asn Asn Asp Asn Gln Leu Asp Gln Phe Pro Arg Arg Phe
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Asn Leu Ala Gly Asn Thr Glu Gln Glu Phe Leu Arg Tyr Gln Gln Gln
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Ser Arg Gln Ser Arg Arg Arg Ser Leu Pro Tyr Ser Pro Tyr Ser Pro
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Gln Ser Gln Pro Arg Gln Glu Glu Arg Glu Phe Ser Pro Arg Gly Gln
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His Ser Arg Arg Glu Arg Ala Gly Gln Glu Glu Glu Asn Glu Gly Gly
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Asn Ile Phe Ser Gly Phe Thr Pro Glu Phe Leu Glu Gln Ala Phe Gln
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Glu Glu Glu Gly Ala Ile Val Thr Val Arg Gly Gly Leu Arg Ile Leu
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Ser Pro Asp Arg Lys Arg Arg Ala Asp Glu Glu Glu Glu Tyr Asp Glu
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Asp Glu Tyr Glu Tyr Asp Glu Glu Asp Arg Arg Arg Gly Arg Gly Ser
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Arg Gly Arg Gly Asn Gly Ile Glu Glu Thr Ile Cys Thr Ala Ser Ala
 325 330 335

Lys Lys Asn Ile Gly Arg Asn Arg Ser Pro Asp Ile Tyr Asn Pro Gln
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Ala Gly Ser Leu Lys Thr Ala Asn Asp Leu Asn Leu Leu Ile Leu Arg
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Phe Phe Val Pro Pro Ser Gln Gln Ser Pro Arg Ala Val Ala
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☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Scanned copy is best available.